

Published in final edited form as:

Gene. 2014 September 1; 547(2): 239–244. doi:10.1016/j.gene.2014.06.047.

An enhancer- blocking element regulates the cell-specific expression of alcohol dehydrogenase 7

Sowmya Jairam^a and Howard J. Edenberg^{a,b,*}

^aDepartment of Biochemistry and Molecular Biology, Indiana University School of Medicine, 635 Barnhill Drive, MS4063, Indianapolis, IN 46202-5122, United States

^bDepartment of Medical and Molecular Genetics, Indiana University School of Medicine, 635 Barnhill Drive, MS4063, Indianapolis, IN 46202-5122, United States

Abstract

The class IV alcohol dehydrogenase gene *ADH7* encodes an enzyme that is involved in ethanol and retinol metabolism. *ADH7* is expressed mainly in the upper gastrointestinal tract and not in the liver, the major site of expression of the other closely related *ADHs*. We identified an intergenic sequence (iA1C), located between *ADH7* and *ADH1C*, that has enhancer-blocking activity in liver-derived HepG2 cells that do not express their endogenous *ADH7*. This enhancer blocking function was cell- and position-dependent, with no activity seen in CP-A esophageal cells that express *ADH7* endogenously. iA1C function was not specific to the *ADH* enhancers; it had a similar cell-specific effect on the SV40 enhancer. The CCCTC-binding factor (CTCF), an insulator binding protein, bound iA1C in HepG2 cells but not in CP-A cells. Our results suggest that in liver-derived cells, iA1C blocks the effects of *ADH* enhancers and thereby contributes to the cell specificity of *ADH7* expression.

Keywords

Insulator element; *ADH7*; cell specific gene expression; class IV alcohol dehydrogenase; enhancer; CCCTC-binding factor (CTCF)

1. INTRODUCTION

The alcohol dehydrogenases (ADHs) are a family of metalloenzymes that reversibly oxidize various primary and secondary alcohols. The class IV alcohol dehydrogenase, ADH7 (σ -ADH, μ -ADH), efficiently metabolizes ethanol to the known toxin and carcinogen acetaldehyde (Kedishvili et al., 1995, Yin et al., 1990), and is involved in the first-pass metabolism of alcohol (Pond and Tozer, 1984). ADH7 also metabolizes retinol to retinal

© 2014 Elsevier B.V. All rights reserved.

*Correspondence to: Howard J. Edenberg, Ph.D., Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, 635 Barnhill Drive, MS4063, edenberg@iu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(Yang et al., 1994, Yin et al., 2003), the precursor to the anti-carcinogen and signaling molecule retinoic acid (Rhinn and Dollé, 2012, Uzzaman et al., 2011). *ADH7* has been associated with alcohol metabolism (Birley et al., 2009, Birley et al., 2008), alcoholism (Han et al., 2005, Osier et al., 2004), drug dependence (Levrán et al., 2009) and cancers of the upper aero-digestive tract (McKay et al., 2011, Wei et al., 2010). It is therefore important to understand the regulation of *ADH7*.

ADH7 lies at one end of a cluster of *ADH* genes all oriented in the same direction (Edenberg, 2007, Edenberg, 2012, Hurley and Edenberg, 2012), and is unique among the *ADHs* in being expressed mainly in the esophagus and gastric mucosa but not in the liver, the primary site of expression of the other 6 *ADHs* (Engeland and Maret, 1993, Kedishvili et al., 1995). Regulatory elements extending up to -799 bp are active in HeLa, CV-1 monkey kidney, and H4IIE3 rat liver cells (Kotagiri and Edenberg, 1998). The promoter and other regulatory elements in the *ADH7* proximal region extending 12.5 kb upstream from the translation start site are also active in both CP-A esophageal cells that express *ADH7* and HepG2 hepatoma cells that do not, although there are cell-specific differences in the level of transcriptional activity (Jairam and Edenberg, 2014). They are also active in mouse MEF cells (*unpublished data*). This suggests that other, more distant factors contribute to the cell-specific expression of *ADH7*.

Enhancers can activate promoters over long distances (Arnosti and Kulkarni, 2005, Bulger and Groudine, 2011, Chin-Tong and Victor, 2011). Several *ADH* enhancers have been identified that activate more than one *ADH* promoter. The potent FOXA-dependent liver-specific enhancer located upstream of *ADH4* is capable of activating both *ADH4* and *ADH1B* promoters *in vitro* (Pochareddy and Edenberg, 2010). An HNF1-bound enhancer, located 8.5 kb downstream of *ADH7* in the 59.5 kb intergenic region between *ADH1C* and *ADH7*, was both necessary and sufficient for expression of all three class I *ADHs* in the liver (Su et al., 2006). Yet, despite the presence and proximity of functional *ADH* enhancers, *ADH7* is not expressed in the liver, suggesting the possibility of an element that blocks the effect of these enhancers on *ADH7*.

The eukaryotic genome is organized into domains comprised of individual genes or clusters of genes with distinct patterns of expression (Kadauke and Blobel, 2009, Lunyak, 2008). Active and inactive chromatin domains are often in close proximity to one another, and enhancer and silencer elements operate over large distances to regulate the genes in these domains. Insulators or boundary elements function to prevent regulatory elements within a domain from promiscuously activating or suppressing the expression of genes located in adjacent domains (Barkess and West, 2012, Bushey et al., 2008, Moltó et al., 2009). Insulators typically exhibit either one or both of two characteristics 1) “enhancer blocking” function, i.e., when placed between the enhancer and promoter they block enhancers from activating the promoter and 2) “barrier” function of protecting transgenes from position effects. In vertebrates, most known insulators function by binding the CCCTC-binding factor (CTCF), a zinc finger protein that can recognize diverse DNA elements (Holwerda and de Laat, 2013, Phillips and Corces, 2009). CTCF can function as both a transcriptional activator (Vostrov and Quitschke, 1997) and repressor (Filippova et al., 1996), and is

involved in insulation, genomic imprinting and X-chromosome inactivation (Filippova, 2008). CTCF binding is, therefore, a marker for potential regulatory function.

Bioinformatics analyses suggested that a region between the *ADH* class I enhancer and *ADH7* was potentially an insulator element that can prevent the HNF1-bound enhancer from activating *ADH7*. We have analyzed the function of this region in cell systems that replicate the cell-specific pattern of endogenous *ADH7* expression observed *in vivo*: CP-A esophageal cells that express *ADH7* and HepG2 hepatoma cells that do not. We have demonstrated that it can function as an insulator element that can contribute to cell-specific *ADH7* expression.

2. MATERIALS AND METHODS

2.1. Bioinformatics

The insulatordb database (Bao et al., 2008) was used to identify potential CTCF binding sites in the vicinity of *ADH7*. The International HapMap database (International HapMap Consortium, 2010) was used to obtain information on haplotypes and the DNA samples that could be used for cloning specific haplotypes.

2.2. Cloning of test fragments

The rs2851028-A haplotype of the 841 bp *ADH7* promoter (A7P-A, referred to henceforth as A7P; -19 to -859 bp relative to the *ADH7* translation start site; chr4: 100,356,409-100,357,249 in GRCh37/hg19) was cloned into HindIII and BglII sites in the multiple cloning site of the pXP2 luciferase vector (Nordeen S. K., 1988) to drive luciferase expression. Restriction sites for NcoI, Acc65I, NotI and XhoI were included in the forward primer of A7P to facilitate further subcloning. The approximately 240 bp fragment corresponding to the class I specific HNF1-bound enhancer (ENH) (Su et al., 2006) was cloned into PciI and NdeI sites approximately 1 kb upstream of the A7P promoter fragment in pXP2. ENH forward primer contained restriction sites for MluI, BssHII, NruI and the reverse primer had AsiSI, AfeI, NgoMIV restriction sites to facilitate further sub-cloning. The 946 bp test fragment (iA1C; located 2 kb upstream of HNF1 bound enhancer and identified as a potential CTCF binding site *in silico*) and its two sub-fragments 1 and 2 were cloned into the AsiSI and NgoMIV sites, and/or the MluI and NruI sites for the enhancer blocking assays. DNA samples used as templates in PCR amplification of the three naturally occurring haplotypes of iA1C were obtained from the Coriell Institute for Medical Research (Camden, New Jersey, USA). The AC haplotype at SNPs rs1442490 and rs1442489 was used unless otherwise noted.

For tests with non-homologous elements, iA1C was cloned into the pGL3 control vector (Groskreutz et al., 1995) containing the luciferase reporter gene driven by the SV40 promoter, with the SV40 enhancer 2 kb upstream of the SV40 promoter. Lambda DNA of size 1216 bp (7716 to 8931 bp relative to translation start site, GenBank: J02459.1) amplified from the plasmid was used as control. The test and control elements were placed between the promoter and enhancer using the MluI and BglII sites in the multiple cloning site (MCS) immediately upstream of the SV40 promoter.

2.3. Cell culture and Transient transfections

HepG2 human hepatoma cells (HB-8065; ATCC, Manassas, VA) were cultured in MEM (ATCC) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 4 mM glutamine (Thermo Scientific Hyclone, Waltham, MA) and 1X Penicillin and Streptomycin (Thermo Scientific Hyclone) on cell bind surface plates (Corning Inc., Tewksbury, MA; CLS3296) at 37°C. Transient transfections in HepG2 cells were done by seeding 0.8×10^5 cells per well in Corning cell bind surface 12-well plates (Corning Inc., Tewksbury, MA; CLS3336). Medium was changed 24 h after seeding, and cells were transfected with 1.52 pmoles of test DNA, 15 ng of pCMV β -galactosidase plasmid (Clontech, Mountain View, CA) and enough pUC19 DNA to get a total DNA amount of 1000 ng per well. Fugene HD (Roche, Indianapolis, IN) was used at 2 μ l per well for the transfection reagent.

CP-A (ATCC, CRL-4027) is an hTERT immortalized cell line obtained from a non-dysplastic Barrett's Esophagus tissue. CP-A cells were cultured in Keratinocyte-SFM (Invitrogen, Carlsbad, CA; 17005-042) at 37 °C. Each 500 ml was supplemented with 25 mg Bovine pituitary extract and 2.5 μ g human recombinant epidermal growth factor supplied with the medium plus 10% FBS and 1% Penicillin-Streptomycin (ATCC, 30-2300). For transient transfections, CP-A cells were seeded at 3.75×10^5 cells per well in 12-well cell bind plates (Corning Inc., Tewksbury, MA; CLS3336). Transfections were done 24 h after seeding as described above.

Luciferase activities were normalized to the internal β -galactosidase control, and the relative activity of each test fragment was measured as the ratio of normalized luciferase value of test construct to that of the corresponding promoter. P-values were calculated using the student's two-tailed t-test.

2.4. Chromatin immunoprecipitation (ChIP)

ChIP assays were done in HepG2 and CP-A cells with Anti-CTCF (Millipore; 07-729), positive control anti-H3 and negative control IgG antibodies supplied with the SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling Technology, Danvers, MA; 9003). Assays were done following the manufacturers protocol, with sonication conditions optimized for the two cell types. CP-A cells were sonicated 3 times for 7 s each and HepG2 cells were sonicated 5 times for 15 s each, with a 60 second interval on ice between sonication bursts. The immunoprecipitations were done overnight at 4 C with gentle rotation, followed by incubation with magnetic beads for 3 h at 4 C. Following DNA elution, quantification was done by qPCR and standard PCR. Eleven primer pairs amplifying overlapping iA1C sub-fragments approximately 100 bp in size were tested for enrichment in the Anti-CTCF IP sample with yields calculated as % input. Human RPL30 primers (Cell Signaling Technology, Danvers, MA; 7014S) specific to exon 3 of the *RPL30* gene, known to be bound by Histone H3 in most cells, served as a positive control for the ChIP protocol with anti-H3 and as a non-specific control for CTCF binding.

2.5. RNA extraction and purification, Real time PCR

HepG2 and CP-A RNA samples were extracted using Trizol and purified using the Qiagen RNeasy mini kit (Qiagen, Germantown, MD; 74106) following the manufacturer's protocol.

RNA concentrations were determined by absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). Superscript III First-strand synthesis system (Invitrogen, Carlsbad, CA; 18080-051) was used to synthesize cDNA from 1 µg RNA following the manufacturer's protocol. qPCR assays were performed using 10 µl of 2X Power SYBR Green mastermix (Life technologies, Carlsbad, CA; 4367659), 2 µl of 3 µM primers, 3 µl of 2X diluted cDNA and water in the StepOnePlus Real-time PCR system (Life Technologies, Carlsbad, CA; 4376598). Controls with no reverse transcription and with no DNA were included in each experiment. For semi-quantitative PCR, 10 µl of Bullseye R-Taq 2X mastermix (Midwest Scientific, MO; BE180301), 2 µl of 5 µM primers, 2 µl of DNA template and water were amplified according to the SimpleChIP Enzymatic Chromatin IP protocol (Cell Signaling Technology, Danvers, MA; 9003). The amplified products were run on a 2% agarose gel with the appropriate controls.

3. RESULTS

3.1. Identification of iA1C, an enhancer blocker with cell-specific function

The class I *ADH* enhancer ENH, located approximately 8.5 kb downstream of the *ADH7* gene, does not activate the *ADH7* promoter in the liver (Su et al., 2006), leading us to hypothesize an enhancer blocking element between the enhancer and *ADH7* promoter. Since vertebrate insulator elements containing enhancer-blocking activity are typically associated with the CCCTC binding factor (CTCF), we searched the insulatordb database (Bao et al., 2008) for potential CTCF binding sites in the vicinity of *ADH7* and found a 946 bp sequence we called iA1C in the intergenic region between the enhancer and *ADH7* (Figure 1A).

To test the potential enhancer blocking activity of the iA1C fragment, we cloned it upstream of the *ADH7* promoter (A7P, with allele A at the functional SNP rs2851028 (Jairam and Edenberg, 2014) on either or both sides of the enhancer ENH (Figure 1B). Constructs were tested for effect on enhancer function in CP-A cells that express endogenous *ADH7* (Jairam and Edenberg, 2014) and in HepG2 cells that do not. ENH increased A7P promoter activity 2.4 fold in CP-A cells and 3.4 fold in HepG2 cells. In CP-A cells, iA1C significantly increased transcriptional activity of the enhancer-containing plasmids independent of its placement with respect to ENH (Figure 2A). In HepG2 cells, iA1C caused a 60% reduction in enhancer activity when placed between ENH and A7P (Figure 2A). When placed outside the enhancer, iA1C had a significant but much smaller effect on activity. Flanking ENH by iA1C on both sides had a dramatic and nearly complete block on ENH function, with the luciferase expression of the flanking construct only slightly higher (1.17 fold) than that of the promoter construct alone. Thus, iA1C had enhancer-blocking activity in HepG2 cells, and this activity was cell-specific and dependent on the placement of iA1C with respect to the enhancer.

To localize the enhancer blocking activity, iA1C was divided into two overlapping shorter fragments of 641 bp and 305 bp and tested for function in HepG2 cells. Enhancer activity was reduced by about 75% when flanked by either sub-fragment (Figure 2B). The full-length iA1C was a more potent enhancer blocker than its sub-fragments, nearly completely eliminating enhancer effect (Figure 2B).

3.2. iA1C function with heterologous elements

To test whether iA1C acted on heterologous regulatory elements, we examined its effects on the SV40 enhancer and SV40 promoter driving luciferase expression in the pGL3 control vector (Groskreutz et al., 1995). The SV40 promoter and enhancer typically work well in nearly all cell types (Schirm et al., 1987). The enhancer increased SV40 promoter activity 180 fold in CP-A cells, and iA1C had little effect on activity (Figure 3A). The enhancer increased SV40 promoter activity 10-fold in HepG2 cells; the AC haplotype of iA1C used in earlier experiments reduced enhancer activity by 44% (Figure 3B). We tested two additional haplotypes of iA1C (Table 1) in this system. The iA1C-GC haplotype had a similar effect while iA1C-GT had a slightly (but significantly) greater effect, reducing enhancer activity by 50% (Figure 3B). The control plasmid containing lambda DNA had little effect on enhancer activity in HepG2 cells indicating the insulator function was specific to iA1C.

3.3. CTCF binds multiple sites within iA1C

Since vertebrate enhancer blockers are typically bound by CTCF, chromatin immunoprecipitation (ChIP) assays were performed in HepG2 cells to identify potential CTCF binding sites within the endogenous iA1C sequence. Of the eleven iA1C sub-fragments (Figure 4A) tested for CTCF binding by ChIP, the greatest binding was seen for fragment D, with the adjacent sub-fragment C also showing high yield (Figure 4B). Another spike in yield was seen for fragment J, indicating multiple binding sites for CTCF within iA1C. Fragment K (adjacent to the iA1C region) and a sequence from exon 3 of *RPL30* tested as a control did not bind CTCF in HepG2 cells, indicating the binding was specific. By contrast, iA1C did not detectably bind CTCF in CP-A cells (Figure 4B).

4. DISCUSSION

In this report, we have identified an intergenic element, iA1C, located between the *ADH7* gene and the class I *ADH* specific enhancer ENH (Su et al., 2006), that blocks ENH activity on the *ADH7* promoter in HepG2 cells. While all chromatin insulators do not have enhancer blocking activity *in vitro*, enhancer blocking sequences identified in the relevant physiological cell systems also function as chromatin insulators *in vivo* (Wallace and Felsenfeld, 2007, Herold et al., 2012). Thus, although transient *in vitro* systems cannot duplicate the full complexity of *in vivo* systems, they are a good indication of insulator function. The enhancer blocking activity was dependent on the placement of iA1C with respect to the enhancer and promoter, acting only when iA1C was placed between the two elements. This function was cell specific, with no enhancer blocking activity in *ADH7*-expressing CP-A cells. Correspondingly, we found cell-specific binding of CTCF to multiple sites within iA1C in HepG2 cells. No binding was seen in CP-A cells, suggesting CTCF binding may be required for iA1C insulator function. Since all known vertebrate insulators bind CTCF (Kim et al., 2007), our finding that CTCF binding to iA1C *in vivo* correlates with its cell specific enhancer blocking activity also supports its function as an endogenous insulator. iA1C showed cell-specificity even on a widely expressed heterologous promoter-enhancer pair from SV40. Thus, the cell-specificity resides within iA1C itself. These findings together strongly suggest that iA1C plays a role in tissue specific

expression of *ADH7*, particularly its lack of expression in the liver, by serving as an insulator element.

ADH7 is divided into 2 distinct Linkage Disequilibrium (LD) blocks, with the recombination hotspot within the gene (Edenberg et al., 2006, Han et al., 2005, Osier et al., 2004). Single nucleotide polymorphisms (SNPs) in the 5' LD block of *ADH7* are associated with alcoholism (Levrán et al., 2009), alcohol metabolism (Birley et al., 2008, Birley et al., 2009), cancers (McKay et al., 2011, Wei et al., 2010) and drug dependence (Levrán et al., 2009). Studies have shown that regulatory elements upstream of *ADH7*, including the promoter, are active in various cell types including HepG2, CV-1, H4IIE3 and CP-A cells (Kotagiri and Edenberg, 1998; Jairam and Edenberg, 2014). Variants of these regulatory elements can affect function depending on cell type and DNA sequence, and are in LD with SNPs associated with alcohol dependence, alcohol metabolism and cancer (Jairam and Edenberg, 2014). The 3' *ADH7* LD block ends in a region intergenic between *ADH7* and *ADH1C*, with little LD with the rest of the *ADH* cluster. iA1C and the class I enhancer fall within the 3' LD block, which has reported disease associations, including rs284787 with cancer of the upper aerodigestive tract (Oze et al., 2009) and rs284786 with personality traits and substance dependence (Luo et al., 2008), but none with the variants of iA1C. One of the common iA1C variants we tested, iA1C-GT, had a slightly stronger effect on insulator function, likely due to the T variant at rs1442489 (C/T). iA1C insulator function and cell-specificity with the potent SV40 enhancer suggests that it protects the *ADH7* promoter from not just the proximal class I *ADH* enhancer, but from other distal enhancers as well.

Chromatin insulators have emerged as important factors in the spatial and topological organization of higher order chromatin structures and functional transcriptional domains (Phillips-Cremens and Corces, 2013, Van Bortle and Corces, 2012). Insulators can employ any one or a combination of mechanisms depending on cell type and target enhancers and promoters (Bushey et al., 2008, Herold et al., 2012, Valenzuela and Kamakaka, 2006, Wallace and Felsenfeld, 2007, Zhu et al., 2007). CTCF can interact with itself forming homodimers, with other regulatory proteins (Merkenschlager and Odom, 2013, Weth and Renkawitz, 2011), and also with the nuclear lamina causing DNA looping and formation of *cis*- and *trans*- chromatin domains and influencing cross talk between gene promoters and regulatory elements (Phillips and Corces, 2009, Williams and Flavell, 2008). Cell specific interactions between CTCF bound sequences are known to regulate the cell specific expression of linked genes such as the β -globin cluster (Junier et al., 2012, Ren et al., 2012). Since our results show that unbound iA1C correlates with a lack of enhancer blocking activity, it is likely that CTCF binding is a necessary event for iA1C function as an insulator.

We have shown that iA1C insulates the *ADH7* gene from the class I *ADH* enhancer ENH, and also the heterologous SV40 enhancer, in liver-derived cells. Thus it should potentially insulate *ADH7* from other enhancers including the potent liver-specific *ADH4* enhancer 4E3 (Pochareddy and Edenberg, 2010) further downstream. We demonstrate that the function of iA1C is cell-specific, as is the binding of CTCF to iA1C. Thus we conclude that the insulator iA1C helps determine the tissue specificity of *ADH7* expression. There is a small but significant difference in function due to genetic variation at rs1442489. Earlier work demonstrated cell specificity and significant effects of genetic variations in several more

proximal elements, including an enhancer 7P10 and the *ADH7* promoter itself (Jairam and Edenberg, 2014). Thus, a combination of factors is important for the overall and precise regulation of *ADH7* transcription.

Acknowledgments

We thank Jun Wang and Ronald E. Jerome for technical assistance. This research was supported by a grant from the National Institute on Alcohol Abuse and Alcoholism R37-AA006460.

ABBREVIATIONS

A7P	<i>ADH7</i> promoter
ADH	alcohol dehydrogenase
CTCF	CCCTC binding factor
ENH	class I <i>ADH</i> enhancer
hTERT	human telomerase reverse transcriptase
LD	Linkage Disequilibrium
SNP	single nucleotide polymorphism

References

- ARNOSTI DN, KULKARNI MM. Transcriptional enhancers: Intelligent enhanceosomes or flexible billboards? *Journal of Cellular Biochemistry*. 2005; 94:890–898. [PubMed: 15696541]
- BAO L, ZHOU M, CUI Y. CTCFBSDB: a CTCF-binding site database for characterization of vertebrate genomic insulators. *Nucleic Acids Research*. 2008; 36:D83–D87. [PubMed: 17981843]
- BARKESS G, WEST AG. Chromatin insulator elements: establishing barriers to set heterochromatin boundaries. *Epigenomics*. 2012; 4:67–80. [PubMed: 22332659]
- BIRLEY AJ, JAMES MR, DICKSON PA, MONTGOMERY GW, HEATH AC, MARTIN NG, WHITFIELD JB. ADH single nucleotide polymorphism associations with alcohol metabolism in vivo. *Human Molecular Genetics*. 2009; 18:1533–1542. [PubMed: 19193628]
- BIRLEY AJ, JAMES MR, DICKSON PA, MONTGOMERY GW, HEATH AC, WHITFIELD JB, MARTIN NG. Association of the gastric alcohol dehydrogenase gene *ADH7* with variation in alcohol metabolism. *Human Molecular Genetics*. 2008; 17:179–189. [PubMed: 17921519]
- BULGER M, GROUDINE M. Functional and Mechanistic Diversity of Distal Transcription Enhancers. *Cell*. 2011; 144:327–339. [PubMed: 21295696]
- BUSHEY AM, DORMAN ER, CORCES VG. Chromatin Insulators: Regulatory Mechanisms and Epigenetic Inheritance. *Molecular cell*. 2008; 32:1–9. [PubMed: 18851828]
- CHIN-TONG O, VICTOR GC. Enhancer function: new insights into the regulation of tissue-specific gene expression. *Nature Reviews Genetics*. 2011; 12:283–293.
- EDENBERG HJ. The genetics of alcohol metabolism: role of alcohol dehydrogenase and aldehyde dehydrogenase variants. *Alcohol Res Health*. 2007; 30:5–13. [PubMed: 17718394]
- EDENBERG HJ. Genes contributing to the development of alcoholism: an overview. *Alc Health Res*. 2012; 34:336–338.
- EDENBERG HJ, XUEI X, CHEN HJ, TIAN H, WETHERILL LF, DICK DM, ALMASY L, BIERUT L, BUCHOLZ KK, GOATE A, HESSELBROCK V, KUPERMAN S, NURNBERGER J, PORJESZ B, RICE J, SCHUCKIT M, TISCHFIELD J, BEGLEITER H, FOROUD T. Association of alcohol dehydrogenase genes with alcohol dependence: a comprehensive analysis. *Human Molecular Genetics*. 2006; 15:1539–1549. [PubMed: 16571603]

- ENGELAND K, MARET W. Extrahepatic, Differential Expression of Four Classes of Human Alcohol Dehydrogenase. *Biochemical and Biophysical Research Communications*. 1993; 193:47–53. [PubMed: 8503936]
- FILIPPOVA GN. Genetics and epigenetics of the multifunctional protein CTCF. *Current topics in developmental biology*. 2008; 80:337–360. [PubMed: 17950379]
- FILIPPOVA GN, FAGERLIE S, KLENOVA EM, MYERS C, DEHNER Y, GOODWIN G, NEIMAN PE, COLLINS SJ, LOBANENKOV VV. An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. *Molecular and Cellular Biology*. 1996; 16:2802–13. [PubMed: 8649389]
- GROSKREUTZ DJ, SHERF BA, WOOD KV, SCHENBORN ET. Increased expression and convenience with the new pGL3 Luciferase Reporter Vectors. *Promega Notes*. 1995:2–8.
- HAN Y, OOTA H, OSIER MV, PAKSTIS AJ, SPEED WC, ODUNSI A, OKONOFUA F, KAJUNA SLB, KAROMA NJ, KUNGULILO S, GRIGORENKO E, ZHUKOVA OV, BONNE-TAMIR B, LU RB, PARNAS J, SCHULZ LO, KIDD JR, KIDD KK. Considerable Haplotype Diversity within the 23kb Encompassing the ADH7 Gene. *Alcoholism: Clinical and Experimental Research*. 2005; 29:2091–2100.
- HEROLD M, BARTKUHN M, RENKAWITZ R. CTCF: insights into insulator function during development. *Development*. 2012; 139:1045–1057. [PubMed: 22354838]
- HOLWERDA SJB, DE LAAT W. CTCF: the protein, the binding partners, the binding sites and their chromatin loops. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 2013; 368
- HURLEY TD, EDENBERG HJ. Genes Encoding Enzymes Involved in Ethanol Metabolism. *Alc Res Health*. 2012; 34:339–44.
- THE INTERNATIONAL HAPMAP CONSORTIUM. Integrating common and rare genetic variation in diverse human populations. *Nature*. 2010; 467:52–58. [PubMed: 20811451]
- JAIRAM S, EDENBERG HJ. Single-Nucleotide Polymorphisms Interact to Affect ADH7 Transcription. *Alcoholism: Clinical and Experimental Research*. 2014; 38:921–929.
- JUNIER I, DALE RK, HOU C, KÉPÈS F, DEAN A. CTCF-mediated transcriptional regulation through cell type-specific chromosome organization in the β -globin locus. *Nucleic Acids Research*. 2012; 40:7718–7727. [PubMed: 22705794]
- KADAUKE S, BLOBEL GA. Chromatin loops in gene regulation. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*. 2009; 1789:17–25.
- KEDISHVILI NY, BOSRON WF, STONE CL, HURLEY TD, PEGGS CF, THOMASSON HR, POPOV KM, CARR LG, EDENBERG HJ, LI TK. Expression and Kinetic Characterization of Recombinant Human Stomach Alcohol Dehydrogenase: ACTIVE-SITE AMINO ACID SEQUENCE EXPLAINS SUBSTRATE SPECIFICITY COMPARED WITH LIVER ISOZYMES. *Journal of Biological Chemistry*. 1995; 270:3625–3630. [PubMed: 7876099]
- KIM TH, ABDULLAEV ZK, SMITH AD, CHING KA, LOUKINOV DI, GREEN ROLANDD, ZHANG MQ, LOBANENKOV VV, REN B. Analysis of the Vertebrate Insulator Protein CTCF-Binding Sites in the Human Genome. *Cell*. 2007; 128:1231–1245. [PubMed: 17382889]
- KOTAGIRI S, EDENBERG HJ. Regulation of Human Alcohol Dehydrogenase Gene ADH7: Importance of an AP-1 Site. *DNA Cell Biol*. 1998; 17:583–590. [PubMed: 9703017]
- LEVRAN O, LONDONO D, O'HARA K, RANDESI M, ROTROSEN J, CASADONTE P, LINZY S, OTT J, ADELSON M, KREEK MJ. Heroin addiction in African Americans: a hypothesis-driven association study. *Genes, Brain and Behavior*. 2009; 8:531–540.
- LUNYAK VV. Boundaries. Boundaries...Boundaries??? *Current Opinion in Cell Biology*. 2008; 20:281–287. [PubMed: 18524562]
- LUO X, KRANZLER HR, ZUO L, ZHANG H, WANG S, GELERNTER J. ADH7 variation modulates extraversion and conscientiousness in substance-dependent subjects. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*. 2008; 147B:179–186.
- MCKAY JD, TRUONG T, GABORIEAU V, CHABRIER A, CHUANG SC, BYRNES G, ZARIDZE D, SHANGINA O, SZESZENIA-DABROWSKA N, LISSOWSKA J, RUDNAI P, FABIANOVA E, BUCUR A, BENCKO V, HOLCATOVA I, JANOUT V, FORETOVA L, LAGIOU P,

TRICHOPOULOS D, BENHAMOU S, BOUCHARDY C, AHRENS W, MERLETTI F, RICHIARDI L, TALAMINI R, BARZAN L, KJAERHEIM K, MACFARLANE GJ, MACFARLANE TV, SIMONATO L, CANOVA C, AGUDO A, CASTELLSAGUÉ X, LOWRY R, CONWAY DI, MCKINNEY PA, HEALY CM, TONER ME, ZNAOR A, CURADO MP, KOIFMAN S, MENEZES A, WÜNSCH-FILHO V, NETO JE, GARROTE LF, BOCCIA S, CADONI G, ARZANI D, OLSHAN AF, WEISSLER MC, FUNKHOUSER WK, LUO J, LUBI SKI J, TRUBICKA J, LENER M, OSZUTOWSKA D, SCHWARTZ SM, CHEN C, FISH S, DOODY DR, MUSCAT JE, LAZARUS P, GALLAGHER CJ, CHANG SC, ZHANG ZF, WEI Q, STURGIS EM, WANG LE, FRANCESCHI S, HERRERO R, KELSEY KT, MCCLEAN MD, MARSIT CJ, NELSON HH, ROMKES M, BUCH S, NUKUI T, ZHONG S, LACKO M, MANNI JJ, PETERS WHM, HUNG RJ, MCLAUGHLIN J, VATTEN L, NJØLSTAD I, GOODMAN GE, FIELD JK, LILOGLOU T, VINEIS P, CLAVEL-CHAPELON F, PALLI D, TUMINO R, KROGH V, PANICO S, GONZÁLEZ CA, QUIRÓS JR, MARTÍNEZ C, NAVARRO C, ARDANAZ E, LARRAÑAGA N, et al. A Genome-Wide Association Study of Upper Aerodigestive Tract Cancers Conducted within the INHANCE Consortium. *PLoS Genet.* 2011; 7:e1001333. [PubMed: 21437268]

MERKENSCHLAGER M, ODOM DT. CTCF and Cohesin: Linking Gene Regulatory Elements with Their Targets. *Cell.* 2013; 152:1285–1297. [PubMed: 23498937]

MOLTÓ E, FERNÁNDEZ A, MONTOLIU L. Boundaries in vertebrate genomes: different solutions to adequately insulate gene expression domains. *Briefings in Functional Genomics & Proteomics.* 2009; 8:283–296. [PubMed: 19752046]

NORDEEN SK. Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques.* 1988; 6:454–8. [PubMed: 2908509]

OSIER MV, LU RB, PAKSTIS AJ, KIDD JR, HUANG SY, KIDD KK. Possible epistatic role of ADH7 in the protection against alcoholism. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics.* 2004; 126B:19–22.

OZE I, MATSUO K, SUZUKI T, KAWASE T, WATANABE M, HIRAKI A, ITO H, HOSONO S, OZAWA T, HATOOKA S, YATABE Y, HASEGAWA Y, SHINODA M, KIURA K, TAJIMA K, TANIMOTO M, TANAKA H. Impact of Multiple Alcohol Dehydrogenase Gene Polymorphisms on Risk of Upper Aerodigestive Tract Cancers in a Japanese Population. *Cancer Epidemiology Biomarkers & Prevention.* 2009; 18:3097–3102.

PHILLIPS JE, CORCES VG. CTCF: Master Weaver of the Genome. *Cell.* 2009; 137:1194–1211. [PubMed: 19563753]

PHILLIPS-CREMINS JE, CORCES VG. Chromatin Insulators: Linking Genome Organization to Cellular Function. *Molecular cell.* 2013; 50:461–474. [PubMed: 23706817]

POCHAREDDY S, EDENBERG HJ. Identification of a FOXA-dependent enhancer of human alcohol dehydrogenase 4 (ADH4). *Gene.* 2010; 460:1–7. [PubMed: 20363298]

POND S, TOZER T. First-Pass Elimination Basic Concepts and Clinical Consequences. *Clinical Pharmacokinetics.* 1984; 9:1–25. [PubMed: 6362950]

REN L, WANG Y, SHI M, WANG X, YANG Z, ZHAO Z. CTCF Mediates the Cell-Type Specific Spatial Organization of the *Kcnq5* Locus and the Local Gene Regulation. *PLoS ONE.* 2012; 7:e31416. [PubMed: 22347474]

RHINN M, DOLLÉ P. Retinoic acid signalling during development. *Development.* 2012; 139:843–858. [PubMed: 22318625]

SCHIRM S, JIRICNY J, SCHAFFNER W. The SV40 enhancer can be dissected into multiple segments, each with a different cell type specificity. *Genes & Development.* 1987; 1:65–74. [PubMed: 2828161]

SU JS, TSAI TF, CHANG HM, CHAO KM, SU TS, TSAI SF. Distant HNF1 Site as a Master Control for the Human Class I Alcohol Dehydrogenase Gene Expression. *Journal of Biological Chemistry.* 2006; 281:19809–19821. [PubMed: 16675441]

UZZAMAN S, GURUVAYOORAPPAN C, BERLIN GRACE VM. All Trans Retinoic Acid and Cancer. *Immunopharmacology and Immunotoxicology.* 2011; 33:241–249. [PubMed: 20929432]

VALENZUELA L, KAMAKAKA RT. Chromatin Insulators*. *Annual Review of Genetics.* 2006; 40:107–138.

- VAN BORTLE K, CORCES VG. Nuclear Organization and Genome Function. Annual Review of Cell and Developmental Biology. 2012; 28:163–187.
- VOSTROV AA, QUITSCHKE WW. The Zinc Finger Protein CTCF Binds to the APB β Domain of the Amyloid β -Protein Precursor Promoter: EVIDENCE FOR A ROLE IN TRANSCRIPTIONAL ACTIVATION. Journal of Biological Chemistry. 1997; 272:33353–33359. [PubMed: 9407128]
- WALLACE JA, FELSENFELD G. We gather together: insulators and genome organization. Current opinion in genetics & development. 2007; 17:400–407. [PubMed: 17913488]
- WEI S, LIU Z, ZHAO H, NIU J, WANG LE, EL-NAGGAR AK, STURGIS EM, WEI Q. A single nucleotide polymorphism in the alcohol dehydrogenase 7 gene (alanine to glycine substitution at amino acid 92) is associated with the risk of squamous cell carcinoma of the head and neck. Cancer. 2010; 116:2984–2992. [PubMed: 20336794]
- WETH O, RENKAWITZ R. CTCF function is modulated by neighboring DNA binding factors. Biochemistry and Cell Biology. 2011; 89:459–468. [PubMed: 21895576]
- WILLIAMS A, FLAVELL RA. The role of CTCF in regulating nuclear organization. The Journal of Experimental Medicine. 2008; 205:747–750. [PubMed: 18347103]
- YANG ZN, DAVIS GJ, HURLEY TD, STONE CL, LI TK, BOSRON WF. Catalytic Efficiency of Human Alcohol Dehydrogenases for Retinol Oxidation and Retinal Reduction. Alcoholism: Clinical and Experimental Research. 1994; 18:587–591.
- YIN S-J, CHOU C-F, LAI C-L, LEE S-L, HAN C-L. Human class IV alcohol dehydrogenase: kinetic mechanism, functional roles and medical relevance. Chemico-Biological Interactions. 2003; 143–144:219–227.
- YIN SJ, WANG MF, LIAO CS, CHEN CM, WU CW. Identification of a human stomach alcohol dehydrogenase with distinctive kinetic properties. Biochem Int. 1990; 22:829–35. [PubMed: 2099148]
- ZHU X, LING J, ZHANG L, PI W, WU M, TUAN D. A facilitated tracking and transcription mechanism of long-range enhancer function. Nucleic Acids Research. 2007; 35:5532–5544. [PubMed: 17704132]

Highlights

- iA1C is a cell-specific insulator with enhancer blocking activity for *ADH7*.
- iA1C functions with homologous and heterologous elements.
- The CCCTC binding factor (CTCF) binds iA1C in the cell type in which iA1C is active, and not in the cell in which it is inactive.
- Genetic variation leads to a small but significant difference in function.

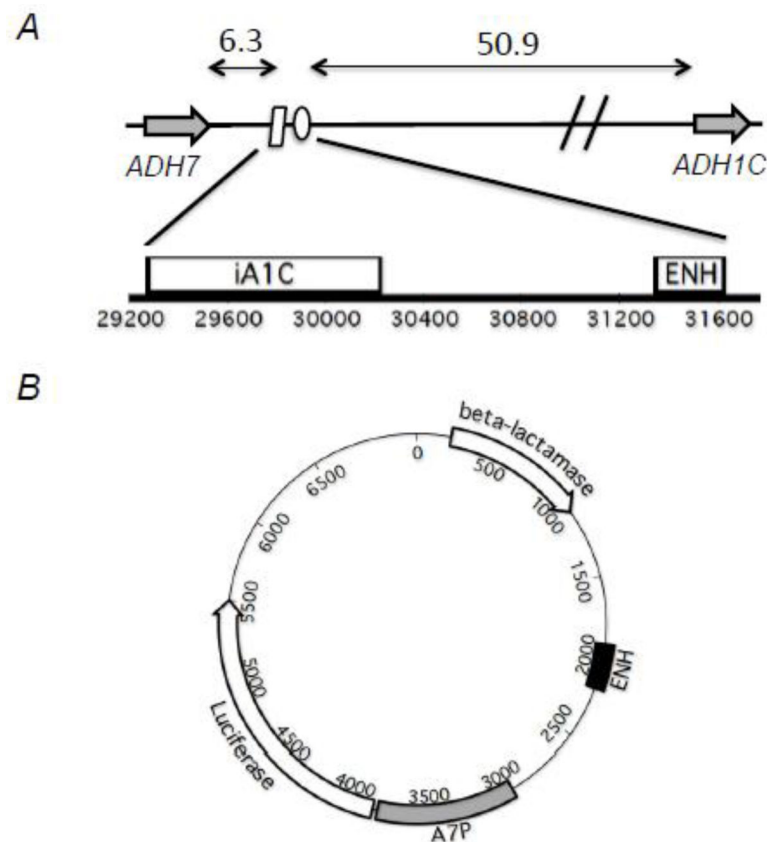
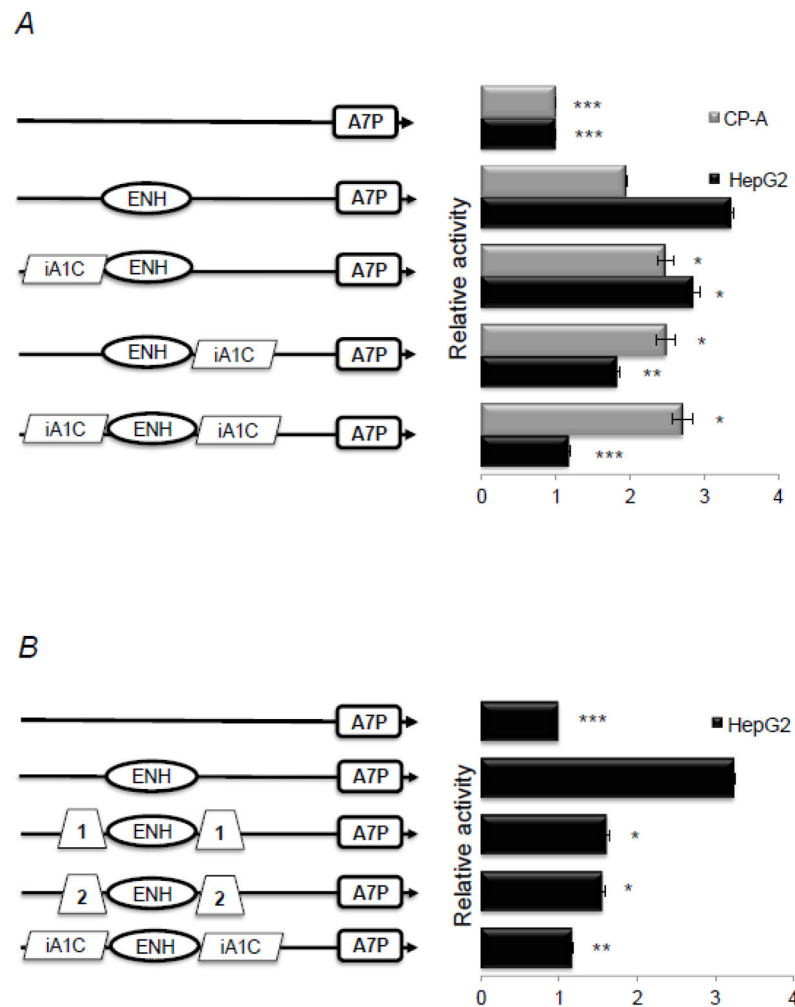


Figure 1.

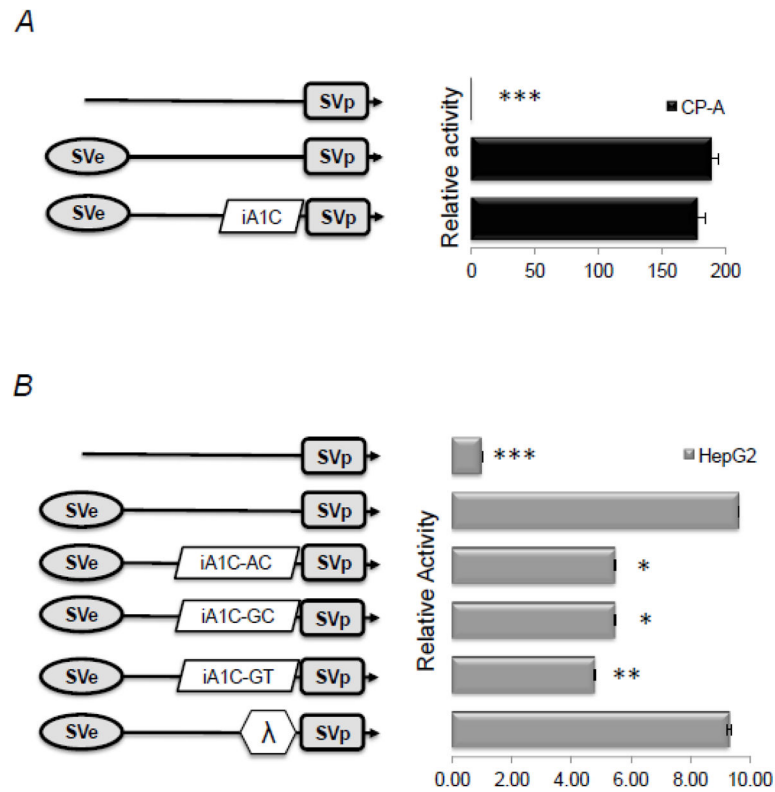
A) Location of the *ADH* class I enhancer (ENH) and *iA1C*. At the top is the part of chromosome 4 containing *ADH7* and *ADH1C*; distances in kb are measured from the ends of *ADH7* and *ADH1C* respectively. Below, *iA1C* and ENH are expanded; distances (bp) are measured from the *ADH7* translation start site (TSS, +1). *iA1C* is the sequence from 29277:30222 with respect to the *ADH7* TSS (chr4:100356390) as +1.

B) Map of the pXP2 plasmid construct used for insulator assays, with the enhancer cloned 1 kb upstream of the *ADH7* promoter A7P. Test fragments were cloned into sites on either side of ENH. Map is drawn to scale, distances in bp.

**Figure 2.**

Cell-specific insulator function. **A)** Effects of iA1C in HepG2 and CP-A cells. Constructs with iA1C cloned on either or both sides of ENH as depicted were transiently transfected into the cells. Relative activities were the ratios of normalized luciferase activity of each construct to that of the promoter (A7P) construct shown at top in the same cell line. Error bars indicate standard errors of the mean. T-tests were done for the differences between the normalized luciferase activity of each construct and the enhancer construct in the corresponding cell type. P-values: * 0.006, ** 5×10^{-6} , *** 8×10^{-8} (n = number of replicates, n = 24).

B) Effects of iA1C sub-fragments iA1C-1 and iA1C-2 containing the sequences from 29277: 29917 and 29918:30222 with respect to *ADH7* TSS. Relative activities in HepG2 cells were determined as the ratio of normalized luciferase activity of each construct to that of the promoter vector. Standard errors of mean are shown (n = 24). P-values reflect the differences between the normalized luciferase activity of each construct and the enhancer construct: * 1×10^{-6} , ** 8×10^{-9} , *** 3×10^{-10} .

**Figure 3.**

iA1C functions with heterologous enhancer and promoter. **A)** Transient transfections in CP-A cells with pGL3 promoter vector (containing the SV40 promoter SVp), pGL3 control vector (containing SVp and the SV40 enhancer SVe), and iA1C cloned between SVp and SVe in the pGL3 control vector. Relative activities represent the ratio of normalized luciferase activity of each construct to that of the pGL3 promoter vector. P-values are the differences between the normalized luciferase activity of each construct and the SVe construct: ** 2.5×10^{-5} , *** 1×10^{-9} (n = 36).

B) Effects of different naturally occurring haplotypes of iA1C and 1.2 kb lambda control (represented as λ) on SV40 enhancer activity in HepG2 cells. iA1C-AC is the haplotype used in earlier assays. Standard errors of mean are shown. P-values represent the differences between the normalized luciferase activity of each construct and SVe construct containing SV40 enhancer: * 5×10^{-5} , ** 1.75×10^{-6} , *** 1×10^{-9} (n = 36).

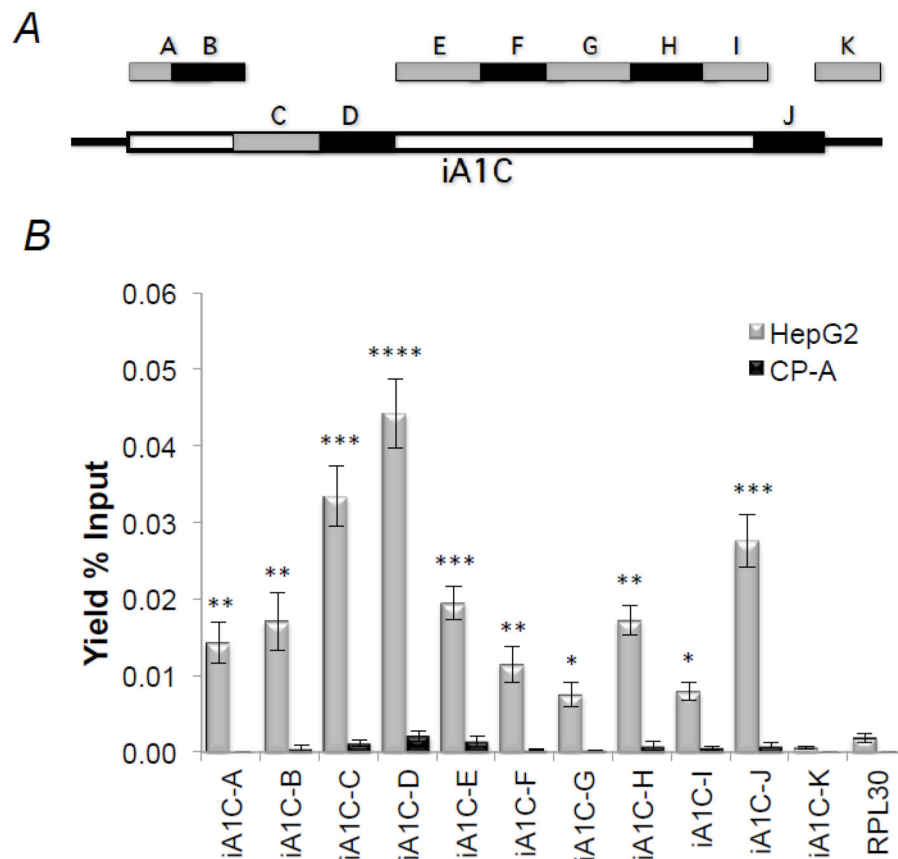


Figure 4.

iA1C binds CTCF cell-specifically. **A**) iA1C subsequences used in Chromatin immunoprecipitation (ChIP) assays. iA1C- A to J are 100–150 bp sequences encompassing the full length of iA1C. K is immediately downstream of iA1C and serves as a control, along with human *RPL30*. The targets C, D and J with the greatest enrichment for CTCF in HepG2 cells are depicted on sequence, with other targets above sequence.

B) CTCF binding to iA1C subsequences in HepG2 and CP-A cells, determined by ChIP. A random sequence (Cell Signaling Technology, Danvers, MA; 7014) in the housekeeping gene *RPL30* serves as a control. Yields = $2^{(\text{Input Ct} - \text{IP Ct})}$, and represent the ratio of signals obtained from ChIP to signals from a 4% input sample, which is the non-immunoprecipitated chromatin sample and serves as a control and as a measure of starting DNA material relative to which the immunoprecipitated chromatin can be measured. Means and standard errors from two or three biological replicates and at least 12 technical replicates are shown. Statistical significance between yields of each target and RPL30 control were calculated by t-tests: * 0.015, ** 0.002, *** 1×10^{-5} , **** 4×10^{-7}

Table 1

The alleles of the two SNPs making up the naturally occurring haplotypes of iA1C, their haplotype frequencies in Northern and Western European populations from Utah (CEU) and the DNA templates from Coriell Institute for Medical Research used for obtaining the sequences are listed.

rs1442490	rs1442489	Haplotype frequency	DNA source
A	C	30.8	NA07000
G	T	63.4	NA12248
G	C	5.8	NA12248